

Grant Title:	<b>Protein Crystal Based Nanomaterials</b>
Type of Report:	<b>Summary of Research – Final Report</b>
Principal Investigator:	<b>Jeffrey A. Bell/Patrick Van Roey</b>
Period Covered by the Report:	<b>June 1, 1997 to November 30, 2001</b>
Recipient's Institution:	<b>Wadsworth Center NYS Department of Health P.O. Box 509, Empire State Plaza Albany, NY 12201-0509</b>
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## Summary of Research Results

### 1. Protein crystals cross-linked to form fibers

Cysteine substitutions were engineered on the surface of maltose binding protein to produce disulfide bonds between adjacent protein molecules in the crystal lattice. The orientations of the disulfide bonds in the crystal lattice were chosen so as to produce one-dimensional cross-links leading to protein fibers for two such maltose binding protein variants.

Only one of the putative fiber-forming proteins (D207C/P316C) produced crystals. The X-ray crystal structure from this variant (Protein Data Bank identifier 1JVX) showed that the cysteine residues were combined with beta-mercaptoethanol present in the crystallization solution to yield mixed disulfides instead of free cysteines or cross-links. The crystals were treated with a reducing agent, followed by air oxidation. X-ray crystal structure determination then demonstrated the formation of the desired cross-links between molecules, with only small changes in the unit cell dimensions (1JVV).

The crystals cross-linked in one dimension were extremely resistant to dissolution in water or neutral buffers. However, they did dissolve in the presence of reducing agents or in dilute acid such as 0.1M acetic acid. Fibers were observed by transmission electron microscopy after negative staining of acidic solutions

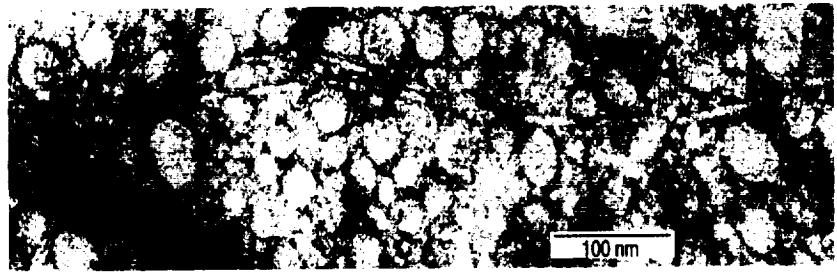


Figure 1. Negatively stained TEM image showing single fibers of cross-linked protein. Two fibers intersect with a set of parallel fibers near the bottom left corner of the micrograph. The upper fiber ends near the center of the image. The circular stain patterns seem to be unrelated to material in the sample.

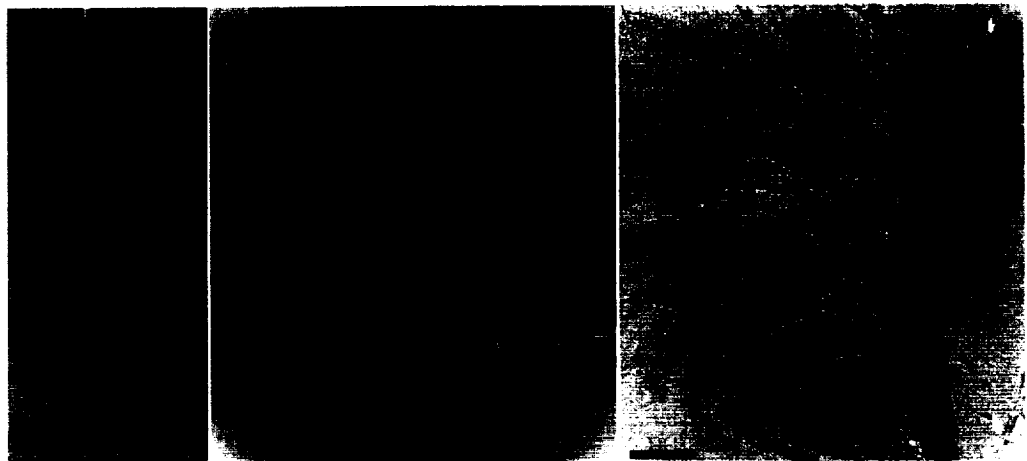


Figure 2. SEM images of a single thread pulled from a droplet of cross-linked protein fibers. The upper end was attached to a cat whisker when the assembly was pulled from the droplet at the bottom of the first micrograph, seen just to the left of the black line. The black line represents 100 microns in the first micrograph, 10 microns in the other two. The object attached to the thread on the right side of the middle panel may be a crystal fragment that did not dissolve in the acidic solution before the droplet was neutralized.

of cross-linked crystals (Fig. 1). The material appeared in micrographs either as individual fibers or as bundles of fibers forming a two-dimensional lattice. The dimension of the spacing along the fiber direction corresponded to the lattice spacing of the crystals in the **b** direction, along which

the disulfides were designed to polymerize, indicating some degree of native-like structure. Fibers were many nanometers in length and would curve gently in the micrographs, but displayed no sharp bends. These fibers are probably related to the acid-dissociated polymers made by cross-linking a variant of T4 lysozyme (Yang *et al.*, 2000). However, those polymers were very short, containing at most 12 monomers. This work demonstrates the synthesis of much longer fibers, containing thousands of monomers, from crystalline protein.

Neutralization of the acidic solutions caused the fibers to associate. Threads composed of many fibers were drawn out of that solution. In scanning electron micrographs, hundreds of individual fibers could be seen unwinding from the ends of some threads. Although much remains to be discovered about these threads and about the physical state of the protein in these assemblies, their appearance is intriguing (Fig. 2).

These observations open the door for the exploration of the properties of a new biomolecular material, derived from protein molecules cross-linked in a known geometry within crystals.

## 2. Engineering of protein to favor crystallization

In the case of a protein that is refractory to crystallization, mutagenesis of surface residues is one possible approach. However, in the absence of a crystal structure, how does one choose which residue to mutate? Statistical analysis shows that the random replacement of lysine residues with arginine should help promote crystallization (Iyer *et al.*, 2000).

To test this recommendation, we chose seven lysines from the sequence of maltose binding protein and substituted them with arginine. These substitutions were K34R, K144R, K179R, K219R, K297R, K305R and K313R. For the purposes of comparison, some other variant proteins were also included in this test. These mutations were I212S, Y341S, F92S and N124Q. These four substitutions were known to be on the surface of the protein from the three-dimensional structure. The first three of this latter set replace a more or less nonpolar residue with serine. N124Q is a very conservative mutation that replaces an asparagine with a glutamine, the difference of one methylene group in the sidechain. Each purified variant protein was incubated at room temperature at 50 different crystallization conditions provided with the Crystal Screen™ kit (Hampton Research) using the hanging drop method. The protein concentration was 8.0 mg/ml in all cases.

The lysine-to-arginine mutations were successful in that two of the seven variants crystallized under conditions where wild type protein would not. In a protein that had not been successfully crystallized before, additional hits in a screen such as this could improve the chances of obtaining diffraction quality crystals.

However, the most successful variant by the above criterion was I212S, which crystallized in two new conditions compared to wild type. Aliphatic hydrophobic residues such as isoleucine are especially disfavored at crystal contacts in the relatively rare instance where they are found on the surface of a protein (Iyer *et al.*, 2000). Therefore, substitution of such nonpolar residues with a more polar residue was expected improve crystallizability. The conservative mutation N124Q also resulted in crystal growth under one new condition. Asparagine is favored at crystal contacts, but glutamine is much more so than asparagine (Iyer *et al.*, 2000).

The conclusion of this study is that the strategy of replacing surface exposed hydrophobic residues with hydrophilic residues would be a useful one if information is available as to which hydrophobic residues are solvent accessible. Lacking such information, as is typically the case, the lysine-to-arginine strategy would be the one to follow, with favorable results to be expected in every one out of three to four substitutions attempted.

## 3. Better knowledge-based potentials for protein-protein contacts

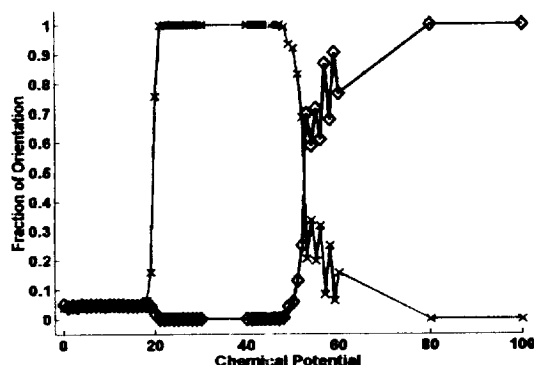
Statistically significant differences were observed in the types of contacts observed for crystallization in low or high ionic strength media. The differences between the types of contacts

observed under these two conditions could most easily be explained by differences in ion binding at protein lattice contacts, illustrating the general importance of ion binding in protein crystallization. Knowledge-based potentials were devised for each of these conditions, in support of the crystallization simulation work described below.

#### 4. Simulation of protein crystallization

A lattice-based Grand Canonical Monte Carlo simulation was developed, in which the protein molecules were modeled as a cluster of rigid united atoms with discrete molecular orientations. The goal was to assess the propensity of any given wildtype or mutant protein to crystallize in any specified space group and unit cell. Interaction energies were derived from knowledge-based potentials and a soft steric repulsion.

An interaction energy test was developed to ensure that the potentials chosen were valid. The design of the test was based on the hypothesis that a protein molecule in a crystalline environment should have a global minimum net protein-protein interaction energy when in the crystallographically observed orientation. The test systematically explored the net interaction energy of a protein molecule in all possible orientations at a fixed location within a crystalline lattice. The global minimum orientation energy for two different proteins was within 5° of the true orientation in the crystal. This observation provided confidence that the potentials were suitable for our protein crystallizability simulation.



**Figure 3.** Fraction of particles in orientations #1 and #22 vs. chemical potential (arbitrary units). Insulin system (1ZNJ), lattice size 9x9x9 unit cells. Line types and symbols: (x) orientation #1; (o) orientation #22. The simulation correctly predicts a broad crystallization slot for the observed orientation (#1) in the crystal, and also predicts the existence of an alternate packing (#22) for the same lattice at very high chemical potential.

The simulation of crystallization investigated the phase behavior of arrays of protein molecules as a function of chemical potential. The dimensions of the lattice and the coordinates of the united atoms were taken directly from the Protein Data Bank file of the system being simulated. The simulation of four protein crystal systems with known crystallization behavior was studied: insulin, maltose binding protein, cutinase and chicken lysozyme. The correct crystalline behavior was observed in three of the systems. The simulations indicated that the proteins studied would exhibit crystallizability in specific ranges of chemical potentials, analogous to a “crystallization slot” describing a range of supersaturation leading to productive crystallization (George and Wilson, 1994). The simulations shown in Figure 3 illustrate this concept, and also make a prediction about an alternate packing of this lattice that might be testable. The simulation was able to correctly rank the anecdotal case of crystallization of lysozyme, insulin, and maltose-binding protein. We also studied the crystallization behavior of four maltose-binding protein mutants. The simulation correctly indicated that the different mutant forms of maltose-binding protein preferred their natural crystal lattice to the lattices of the other maltose-binding protein mutants.

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